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sonic hedgehog and *vascular endothelial growth factor* Act Upstream of the Notch Pathway during Arterial Endothelial Differentiation

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Summary

The appearance of molecular differences between arterial and venous endothelial cells before circulation suggests that genetic factors determine these cell types. We find that *vascular endothelial growth factor* (*vegf*) acts downstream of *sonic hedgehog* (*shh*) and upstream of the Notch pathway to determine arterial cell fate. Loss of Vegf or Shh results in loss of arterial identity, while exogenous expression of these factors causes ectopic expression of arterial markers. Microinjection of *vegf* mRNA into embryos lacking Shh activity can rescue arterial differentiation. Finally, activation of the Notch pathway in the absence of Vegf signaling can rescue arterial marker gene expression. These studies reveal a complex signaling cascade responsible for establishing arterial cell fate and suggest differential effects of Vegf on developing endothelial cells.

Introduction

Arteries and veins can be anatomically distinguished within the adult vertebrate circulatory system (for example, see Gray, 1901). Larger-diameter arterial vessels are exposed to the highest pressure and flow and are characteristically surrounded by a thick medial layer consisting mostly of vascular smooth muscle cells (Gray, 1901; Stehbens, 1995). In contrast, venous vessels carry efferent circulation of lower pressure, have less surrounding smooth muscle, and possess specialized structures, such as valves, to ensure blood flow in a single direction. Although differences in fluid dynamics within the circulatory system play a role in determining the characteristic structure of an artery or vein (Stehbens, 1995), recent evidence suggests that the identity of endothelial cells lining these vessels is established before the onset of circulation by genetic mechanisms during embryonic development (Adams et al., 1999; Gerety et al., 1999; Lawson et al., 2001; Wang et al., 1998).

Molecular differences between arterial and venous endothelial cells have been demonstrated during embryonic development in chick, mouse, and zebrafish. In all of these vertebrate organisms, the transmembrane ligand ephrin-B2 is expressed in endothelial cells lining only arteries, while, in most cases, its receptor, EphB4, is expressed predominantly in venous endothelial cells (Lawson et al., 2001; Moyon et al., 2001; Wang et al., 1998). Artery-restricted expression of ephrin-B2 has also been demonstrated in adult mice and humans (Gale et al., 2001; Shin et al., 2001). Analyses of mice lacking ephrin-B2 (Adams et al., 1999; Wang et al., 1998) or EphB4 (Gerety et al., 1999), as well as *Xenopus laevis* embryos injected with a dominant-negative form of EphB4 (Helbling et al., 2000), show that these factors are required for normal blood vessel development. However, they do not appear to be required for the determination of arterial or venous endothelial cell fate (Wang et al., 1998), suggesting the action of other upstream factors.

We have previously shown that the Notch signaling pathway plays an important role in the expression of artery-specific genes and repression of venous markers within developing arteries (Lawson et al., 2001). Zebrafish *notch5* is expressed within the dorsal aorta (DA), concomitant with the appearance of *ephrin-B2a*, but is not found in veins. (We previously referred to zebrafish *notch5* as *notch3* due to similarities to mouse and human *NOTCH3*. Kortschak et al. (2001) have suggested that the name *notch5* is more appropriate). Zebrafish embryos mutant for the neurogenic gene *mindbomb* (*mib*; Jiang et al., 1996; Schier et al., 1996) exhibit defects in Notch signaling (Appel et al., 1999; Riley et al., 1999) and display ectopic vein marker gene expression within the DA (Lawson et al., 2001). Conversely, endothelial-specific expression of an activated form of *notch5* can repress *flt4* levels in veins (Lawson et al., 2001). Embryos lacking Notch activity also fail to express *ephrin-B2a* and *notch5* within the DA. However, *mib* mutant embryos do exhibit expression of some arterial markers, suggesting that other factors act in parallel to the Notch pathway to induce arterial differentiation.

Among the potential molecules that may act upstream of the Notch pathway to induce arterial differentiation are members of the Hedgehog family of proteins (reviewed in Murone et al., 1999). Several studies suggest an important role for sonic hedgehog (Shh), in particular, during blood vessel development. Zebrafish embryos that are mutant for *sonic you* (*syu*), which encodes the zebrafish homolog of Shh (Schauerte et al., 1998), fail to exhibit normal trunk circulation (Chen et al., 1996). Consistent with these observations, *syu* mutant embryos display defects in the formation of the DA and posterior cardinal vein (PCV), although the initial development of vascular cells appears normal, as indicated by the expression of the endothelial cell marker *fli1* (Brown et al., 2000). Furthermore, recent work has shown that Shh can promote angiogenic blood vessel growth in part by inducing the expression of *vascular endothelial growth factor* (*vegf*) as well as *Angiopoietin-1* and *-2* (Pola et al., 2001). These observations suggest that Shh may cooperate with vascular-specific growth factors during the development of the embryonic vasculature.

In this study we show that endothelial cells in zebrafish embryos lacking Shh activity fail to undergo arterial differentiation, as defined by the expression of artery-specific markers, such as *ephrin-B2a*, while injection of

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mRNA encoding Shh can induce ectopic vascular expression of *ephrin-B2a*. We further show that embryos lacking Shh activity fail to express *vegf* within their somites, and exogenous addition of Vegf in these embryos can rescue vascular *ephrin-B2a* expression, indicating that Vegf is sufficient downstream of Shh to induce arterial differentiation. Consistent with these observations, reduction of Vegf activity using antisense morpholino oligonucleotides phenocopies the arterial differentiation defect found in embryos lacking Shh activity. Finally, we show that Vegf is unable to rescue artery marker gene expression in embryos lacking Notch function, while exogenous Notch activity can induce arterial differentiation in the absence of Vegf signaling. Together with our previous work (Lawson et al., 2001), these observations describe a signaling cascade that is responsible for inducing arterial differentiation and suggests that endothelial cell progenitors respond differentially to Vegf during this process.

Results

sonic hedgehog Is Required for Arterial Differentiation

To investigate the potential role of the Shh pathway during arterial endothelial cell differentiation, we first determined the vascular pattern of *ephrin-B2a* mRNA expression associated with loss of *shh* function in the zebrafish *you*-type class of mutants (van Eeden et al., 1996). We utilized embryos carrying null mutations for *sonic-you* (*syu*) or *you-too* (*yot*), which encode the zebrafish orthologs of Shh and Gli2, a downstream effector of Hh signaling, respectively (Karlstrom et al., 1999; Schauerte et al., 1998). In wild-type sibling embryos derived from an incross of heterozygous syu^{t4} adults, normal expression of *ephrin-B2a* is seen in the DA at 26 hr post fertilization (hpf; Figure 1B). In contrast, embryos mutant for syu^{t4} (Figure 1C) or yot^{ty119} (Figure 1D) fail to express *ephrin-B2a* within their vasculature. Vascular *ephrin-B2a* is also absent when wild-type embryos are exposed to 25 μM cyclopamine (Figure 1E), a potent inhibitor of Shh signaling (Cooper et al., 1998; Incardona et al., 1998; Neumann et al., 1999). Previous studies have shown that endothelial cells are present in *you*-type mutants, as indicated by the expression of *fli1* (Brown et al., 2000). Furthermore, we find that these cells form a single lumenized vessel that remains positive for the venous marker *flt4* in yot^{ty119} or syu^{t4} mutant embryos (Figure 1F and data not shown).

To determine whether Shh is sufficient to induce arterial differentiation, we injected one- to two-cell stage wild-type zebrafish embryos with mRNA encoding Shh and assayed for expression of *ephrin-B2a* transcript. Embryos that were uninjected or injected with mRNA encoding β-galactosidase (β-gal) exhibit expression of *ephrin-B2a* within the DA, but not in the PCV (Figure 1G; 79 out of 79 embryos in two experiments). Embryos injected with β-gal and 25 pg of *shh* mRNA exhibit expanded expression of *ephrin-B2a* into the region of the PCV and ectopic formation of lumenized *ephrin-B2a*-positive vessels (Figure 1H; 40 out of 76 embryos in two representative experiments).

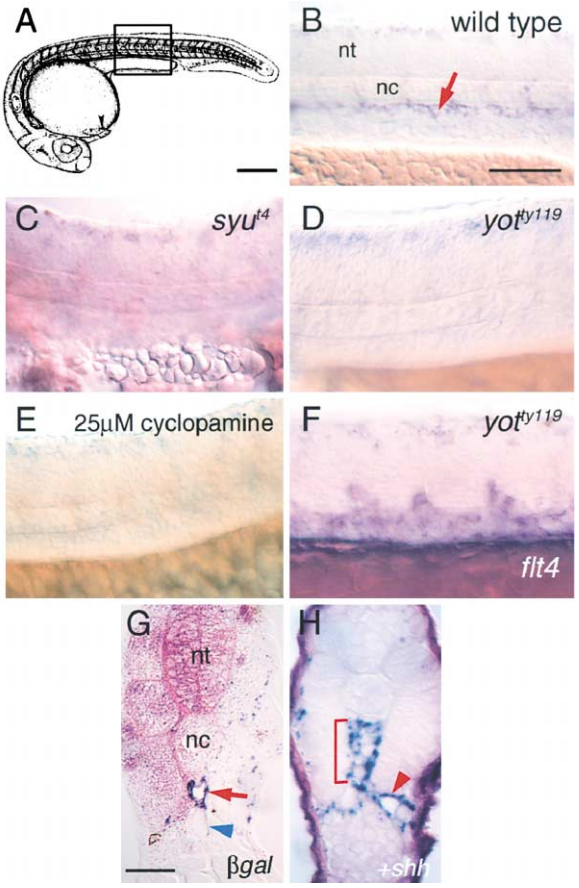

Figure 1. *sonic hedgehog* Is Required for Arterial Differentiation

(A) Line drawing of a zebrafish embryo at approximately 24 hr post fertilization (hpf); modified from Kimmel et al. (1995). Lateral view; anterior is to the left; dorsal is up; scale bar is 250 μm. Boxed region indicates views for (B)–(F).

(B–E) Whole-mount in situ hybridization of *ephrin-B2a* mRNA expression within the trunks of zebrafish embryos at 26 hpf; lateral views; anterior is to the left; dorsal is up; scale bar is 100 μm.

(B) Expression of *ephrin-B2a* in the DA (indicated by red arrow) of a wild-type sibling; nc, notochord; nt, neural tube.

(C and D) Lack of *ephrin-B2a* expression in embryos mutant for (C) syu^{t4} or (D) yot^{ty119}.

(E) Embryo at 24 hpf following treatment with 25 μM cyclopamine; note the lack of *ephrin-B2a* expression.

(F) *flt4* expression in a yot^{ty119} embryo.

(G and H) Cross-sections of the trunks of zebrafish embryos injected with (G) 12.5 pg β-galactosidase (β-gal) or (H) 12.5 pg β-gal and 25 pg *shh* mRNA; dorsal is up; scale bar is 50 μm.

(G) Normal *ephrin-B2a* expression in the DA (indicated by red arrow), but not in PCV (blue arrowhead).

(H) Expanded *ephrin-B2a* expression (red bracket) into the region of the PCV and lumenized ectopic arterial vessel (arrowhead) are apparent in *shh* mRNA-injected embryos.

vegf Rescues Arterial Differentiation in the Absence of *shh* Signaling

Recent work in mouse has shown that Shh can modulate angiogenesis by inducing the expression of *vegf* mRNA (Pola et al., 2001). To determine whether this was the case during zebrafish development, we assayed *vegf* transcript levels in embryos lacking Shh activity. At the 15-somite stage (ss), wild-type sibling embryos express

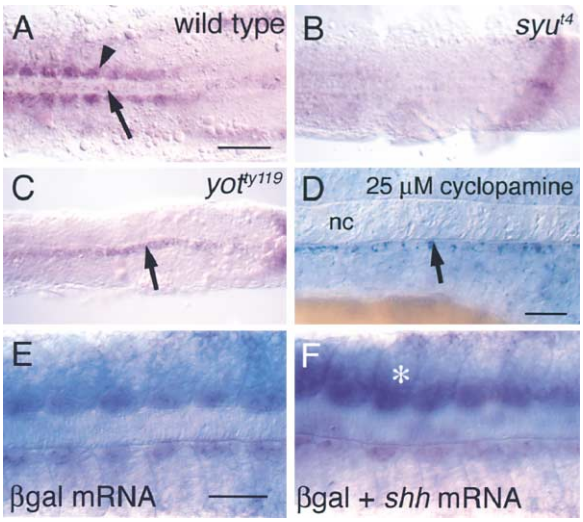

Figure 2. *shh* Controls Somite Expression of *vegf*

(A–C, E, and F) Flat mounts of embryos following whole-mount in situ hybridization for *vegf* mRNA at the 15 ss; dorsal views of the trunk; anterior is to the left.

(D) Lateral view of an embryo following whole-mount in situ hybridization for *vegf* mRNA at 24 hpf; anterior is to the left; dorsal is up; scale bars are (A–C) 250 μm and (D–F) 50 μm.

(A) Wild-type embryo; arrow indicates expression within the hypochord, which is located between the somites; arrowhead indicates somite expression.

(B) *syu*^{t4} mutant embryo lacking somite and hypochord *vegf* expression.

(C) *yot*^{ty119} mutant embryo; note hypochord expression (arrow), but lack of somite expression.

(D) Twenty-four hour post fertilization embryo following exposure to 25 μM cyclopamine; arrow indicates hypochord expression; nc, notochord.

(E) Embryo injected with 12.5 pg β-gal tracer mRNA.

(F) Embryo injected with 12.5 pg β-gal and 25 pg *shh* mRNA; asterisk indicates injected side.

vegf within the medial aspect of the somites, as previously documented (Liang et al., 1998), and in the hypochord (Figure 2A), a structure located between the somites and ventral to the notochord in amphibian and fish embryos (Cleaver et al., 2000; Eriksson and Lofberg, 2000). Embryos mutant for *syu*^{t4} fail to express *vegf* mRNA in the hypochord or somites (Figure 2B). In *yot*^{ty119} mutant embryos (Figure 2C) and wild-type embryos treated with 25 μM cyclopamine (Figure 2D), *vegf* expression is lost within the somites but persists within the hypochord.

To determine whether Shh could also induce expression of *vegf* transcript, we injected mRNA encoding Shh into two- or four-cell stage embryos and assayed for the expression of *vegf* at the 15 ss. Wild-type embryos injected with only β-gal mRNA show normal expression of *vegf* within the somites (Figure 2E; 78 out of 78 embryos in two experiments) and hypochord (data not shown; the hypochord is not visible in the focal plane imaged), while embryos injected with 25 pg of *shh* mRNA exhibit upregulation of *vegf* within the somites (Figure 2F; 75 out of 94 embryos in two representative experiments).

The concomitant loss of *ephrin-B2a* and *vegf* expression in embryos lacking Shh activity suggests the possibility that *vegf* may provide a signal for arterial differentiation downstream of Shh. Therefore, we determined whether exogenous Vegf was sufficient to rescue *ephrin-B2a* expression in the absence of Shh. In most vertebrate embryos, including zebrafish (Liang et al., 2001), two major Vegf isoforms are present: a 121-amino acid isoform ($Vegf_{121}$) that is freely diffusible and a 165-amino acid protein ($Vegf_{165}$) that is associated with the extracellular matrix (reviewed in Robinson and Stringer, 2001). We injected mRNA encoding enhanced green fluorescent protein (EGFP), $Vegf_{121}$, or $Vegf_{165}$ into embryos lacking Shh activity and assayed expression of *ephrin-B2a* at 25 hpf. Embryos injected with 50 pg of EGFP mRNA and treated with ethanol vehicle exhibit normal DA expression of *ephrin-B2a* (data not shown), while those treated with 25 μM cyclopamine failed to express *ephrin-B2a* (Figure 3A; 156 out of 159 embryos in three experiments). Injection of 50 pg of mRNA encoding $Vegf_{121}$ into embryos subsequently exposed to 25 μM cyclopamine rescues expression of *ephrin-B2a* within the trunk (Figure 3B; 74 out of 127 embryos in three experiments). In addition, we injected embryos derived from an incross of *yot*^{ty119} heterozygous adults with mRNA encoding either $Vegf_{121}$ (50 pg) or $Vegf_{165}$ (200 pg). Wild-type siblings injected with either isoform display expanded regions of *ephrin-B2a* expression (Figures 3C and 3E). Uninjected *yot*^{ty119} mutant sibling embryos fail to express *ephrin-B2a* within the trunk vasculature (for example, see Figure 1D; 21 out of 21 mutant embryos in two experiments). We find that *yot*^{ty119} mutant embryos injected with mRNA encoding either $Vegf_{121}$ (Figure 3D; 32 out of 35 mutant embryos in two experiments) or $Vegf_{165}$ (Figure 3F; 41 out of 41 mutant embryos in two experiments) display *ephrin-B2a* expression within the trunk vessels.

Recent data indicate that angioblasts are restricted to an arterial or venous cell fate at midsomitogenesis (the 7–12 ss) in zebrafish embryos (Zhong et al., 2001). We find that, consistent with a role for Vegf during this process, *vegf* mRNA is expressed within the somites at the 10 ss (Figure 3G). In addition, double in situ hybridization with riboprobes against *vegf* and its receptor, *flk1*, show that migrating angioblasts are in close proximity to the source of Vegf at this time (Figure 3H).

vegf Is Required for Arterial Differentiation

Our results indicate that Vegf is sufficient downstream of Shh to induce arterial differentiation and suggest that the loss of *ephrin-B2a* expression in *you*-type mutants is due to the loss of Vegf signaling. To determine whether Vegf is required for arterial differentiation, we utilized antisense morpholino oligonucleotides (Nasevicius and Ekker, 2000) to reduce Vegf activity in zebrafish embryos. Embryos injected with 10 ng of control morpholino display normal expression of *ephrin-B2a* mRNA in the DA at 26 hpf (Figure 4A; see Supplemental Table S1 at http://www.developmentalcell.com/cgi/content/full/3/1/127/DC1), while embryos injected with the same amount of Vegf morpholino exhibit a severe reduction or loss of arterial *ephrin-B2a* expression (Figure 4B; see

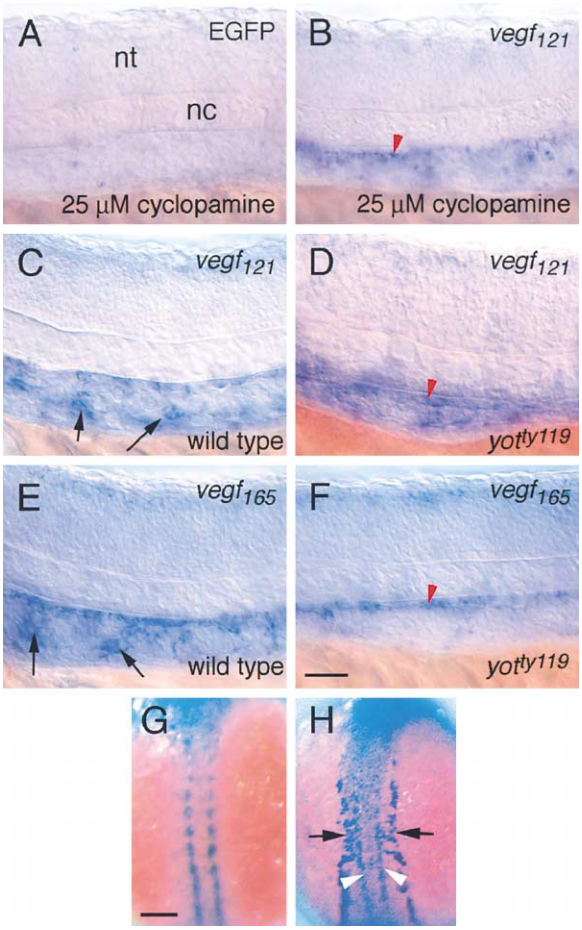

Figure 3. Vegf Rescues Arterial Differentiation in the Absence of Shh Signaling

(A–F) Whole-mount in situ hybridization for *ephrin-B2a* expression at 24 hpf; lateral views; anterior is to the left; dorsal is up; scale bar is 50 μm.

(A) Embryo injected with 50 pg EGFP mRNA and exposed to 25 μM cyclopamine; nc, notochord, nt, neural tube.

(B) Embryo injected with 50 pg of *vegf*$_{121}$ mRNA and treated with 25 μM cyclopamine; red arrowhead indicates presence of *ephrin-B2a*-positive cells in the position of the DA.

(C) Ectopic *ephrin-B2a* expression (indicated by arrows) in a wild-type embryo injected with 50 pg *vegf*$_{121}$ mRNA.

(D) Arrowhead indicates *ephrin-B2a* expression in a *yot*^{ty119} mutant embryo injected with 50 pg of *vegf*$_{121}$ mRNA.

(E) Wild-type sibling injected with 200 pg of mRNA encoding Vegf$_{165}$ displays ectopic *ephrin-B2a* expression in the region of the PCV (arrows).

(F) A *yot*^{ty119} mutant embryo injected with *vegf*$_{165}$ mRNA displays *ephrin-B2a* expression in the region of the DA (indicated by red arrowhead).

(G and H) Dorsal views of ten-somite stage embryos; anterior is up; scale bar is 250 μM.

(G) Expression of *vegf* mRNA within the somites.

(H) Double staining for *vegf* mRNA (white arrowheads) and *flk1*, which is expressed in migrating angioblasts (black arrows) at this time.

Supplemental Table S1). To determine the effects on venous cell fate, we assayed for expression of *flt4*, which is initially expressed in both arterial and venous primordia but becomes restricted to venous vessels by 24 hpf (Thompson et al., 1998). Embryos injected with control morpholino show normal downregulation of *flt4* in the

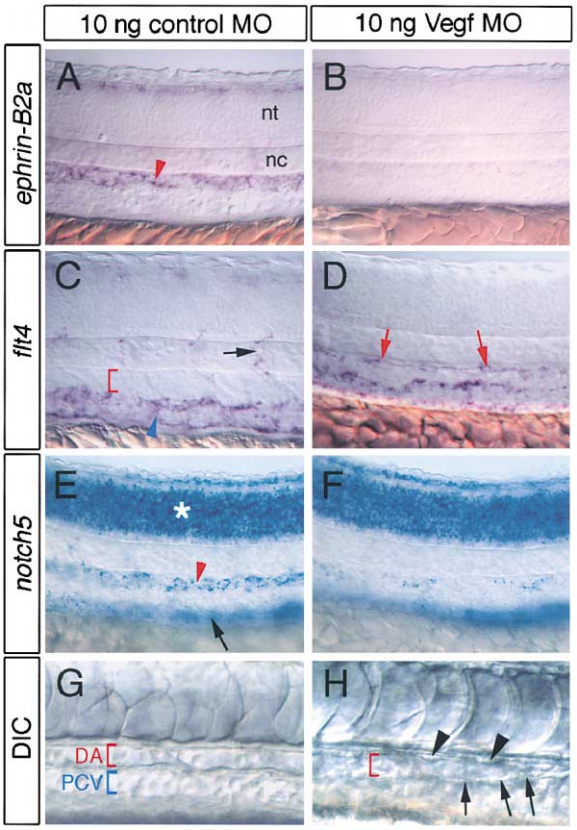

Figure 4. Loss of Vegf Function Perturbs Arterial-Venous Differentiation

(A–F) Whole-mount in situ hybridization of zebrafish embryos at 26 hpf; lateral views; dorsal is up; anterior is to the left; scale bar is 50 μm.

(A) Normal *ephrin-B2a* expression in DA (red arrowhead) of embryo injected with control morpholino; nc, notochord, nt, neural tube.

(B) Loss of DA *ephrin-B2a* expression in an embryo injected with 10 ng of Vegf morpholino.

(C) PCV-restricted *flt4* expression (blue arrowhead) in control morpholino-injected embryos (black arrow indicates normal expression within a segmental vessel); note the lack of expression in the region of the DA (indicated by red bracket).

(D) Ectopic *flt4* expression in the DA in Vegf morpholino-injected embryos. Red arrows indicate expression of *flt4* in cells of the dorsal roof of the DA.

(E) *notch5* expression in DA (red arrowhead) of an embryo injected with control morpholino; white asterisk indicates neural tube and black arrowhead shows pronephric expression outside the focal plane.

(F) Embryo injected with 10 ng of Vegf morpholino; note the decrease in DA *notch5* expression, but no effect on neural tube or pronephric duct expression.

(G and H) Differential interference contrast (DIC) images of live *alb*^{b4} zebrafish embryos at 36 hpf. Lateral views; dorsal is up; anterior is to the right; scale bar is 25 μm

(G) Embryo injected with 10 ng control morpholino; lumens of the DA (red bracket) and PCV (blue bracket) are indicated.

(H) Embryo injected with 10 ng Vegf morpholino. Lumen of the DA is indicated by red bracket; an abnormal narrowing of the DA can be seen (indicated by black arrows). Note the presence of cells with endothelial morphology (black arrowheads) in close proximity to the notochord.

DA by 26 hpf (Figure 4C; see Supplemental Table S1), whereas injection of Vegf morpholino results in persistent expression of *flt4* in the DA (Figure 4D; see Supplemental Table S1). Since the patterns of *ephrin-B2a* and *flt4* expression in embryos injected with Vegf morpholino were similar to those in embryos lacking Notch activity (Lawson et al., 2001), we assayed for arterial expression of *notch5* mRNA. Expression of *notch5* is detected in the DA, pronephric duct, and neural tube of wild-type embryos injected with the control morpholino (Figure 4E; see Supplemental Table S1). In contrast, injection of 10 ng of Vegf morpholino results in a specific loss of *notch5* expression from the DA, while neural tube and pronephric expression is not affected (Figure 4F; see Supplemental Table S1). We observe similar results using a second morpholino directed to the *vegf* 5′ untranslated region (Vegf A-3; Nasevicius et al., 2000; see Supplemental Table S1; data not shown).

We find defects in vascular morphology and circulatory patterns in Vegf morpholino-injected embryos, consistent with perturbation of arterial and venous identity. Embryos injected with 10 ng of control morpholino exhibit normal vascular morphology, as indicated by fully lumenized, well-defined trunk vessels (Figure 4G) and normal circulatory patterns (see Supplemental Table S1). In contrast, zebrafish embryos injected with 10 ng of Vegf morpholino display a disorganized trunk vessel plexus and poorly defined boundaries between the DA and PCV, although endothelial cells can be seen immediately ventral to the notochord, indicating that their migration and localization is not affected (Figure 4H). Consistent with these morphological defects, Vegf morpholino-injected embryos often fail to exhibit trunk or head circulation (see Supplemental Table S1), as has been previously documented (Nasevicius et al., 2000). In addition, we find that a proportion of embryos injected with the Vegf morpholino (see Supplemental Table S1) display arterial-venous shunts between the DA and PCV, similar to those we have previously described in embryos lacking Notch function (Lawson et al., 2001). Embryos injected with 10 ng of Vegf morpholino do not exhibit overt morphological defects at 24–26 hpf and display normal development of the notochord and somites (data not shown and Nasevicius et al., 2000).

Notch Signaling Mediates Arterial Differentiation Downstream of *vegf*

The loss of arterial marker gene expression and ectopic *flt4* transcripts in the DA in Vegf morpholino-injected embryos is similar to that seen in embryos with defective Notch signaling (Lawson et al., 2001), suggesting that *vegf* and the Notch pathway act in a common signaling cascade to induce arterial differentiation. It is also possible that *vegf* expression may be absent in embryos lacking Notch activity, as is the case in embryos lacking Shh (see above). To determine whether *vegf* is affected by Notch signaling, we assayed for its expression in *mib*^{ta52b} mutant embryos. Although the product of the *mib* gene is unknown, many of the phenotypes in *mib*^{ta52b} mutant embryos are similar to those in embryos mutant for the Notch ligand deltaA (Appel et al., 1999). Furthermore, we have previously demonstrated that the vascular defects associated with *mib*^{ta52b} can be phenocopied using a dominant-negative inhibitor of Notch signaling (Lawson et al., 2001). Wild-type siblings exhibit normal expression of *vegf* mRNA within the somites (Figure 5A) and

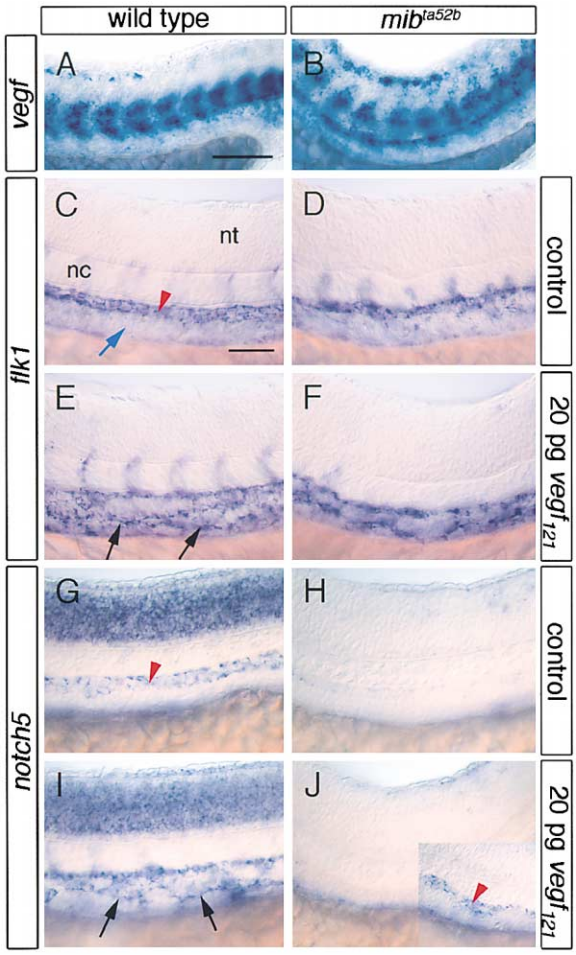

Figure 5. Interaction of the Notch Signaling Pathway and *vegf*

(A–J) Lateral views; anterior is to the left; dorsal is up; embryos at 25 hpf.

(A and B) Whole-mount in situ hybridization for *vegf* mRNA; scale bar is 100 μm.

(A) Wild-type sibling.

(B) Embryo mutant for *mib*^{ta52b}.

(C) *flk1* expression in an uninjected wild-type sibling embryo. Note the higher expression of *flk1* within the DA (red arrowhead) than the PCV (blue arrow); nc, notochord, nt, neural tube.

(D) Uninjected *mib*^{ta52b} mutant embryo with *flk1* expression similar to its wild-type sibling in (C).

(E) Wild-type sibling injected with 20 pg mRNA encoding Vegf$_{121}$; note the increased *flk1* expression in PCV (black arrows).

(F) *mib*^{ta52b} mutant embryo injected with 20 pg of mRNA encoding Vegf$_{121}$ also expresses *flk1* throughout the vasculature.

(G) *notch5* expression in a wild-type sibling embryo injected with EGFP mRNA. DA expression is indicated by red arrowhead.

(H) Lack of *notch5* expression in embryo mutant for *mib*^{ta52b} and injected with EGFP mRNA.

(I) Wild-type sibling injected with 20 pg of *vegf*$_{121}$ mRNA; note the ectopic *notch5* expression within PCV (black arrows).

(J) Mutant *mib*^{ta52b} embryo injected with 20 pg of *vegf*$_{121}$ mRNA showing lack of *notch5* expression. Inset shows rare induction (see Supplemental Table S2 at http://www.developmentalcell.com/cgi/content/full/3/1/127/DC1) of *notch5* expression in DA.

(C–J) Scale bar is 50 μm.

hypochord (data not shown) at 24 hpf. Consistent with somite boundary defects in *mib*^{ta52b} mutant embryos (Jiang et al., 1996), the pattern of *vegf* expression appears to be affected (Figure 5B), although *vegf* mRNA levels are relatively normal compared to those in embryos lacking Shh function (compare Figure 5B and Figures 2B–2D).

In addition to *vegf*, we investigated the expression of the zebrafish Vegf receptor-2 homolog *flk1* in *mib*^{ta52b} mutant embryos. As shown previously (Liao et al., 1997), *flk1* is expressed at higher levels within the DA than within the PCV in wild-type sibling embryos at 24 hpf (Figure 5C) and remains normal in embryos mutant for *mib*^{ta52b} (Figure 5D; see Supplemental Table S2 at http://www.developmentalcell.com/cgi/content/full/3/1/127/DC1). Recent evidence in zebrafish has shown that *flk1* is upregulated throughout the vasculature by Vegf, resulting in ectopic expression within the PCV (Liang et al., 2001). We determined whether this response to *vegf* required intact Notch signaling. In wild-type sibling embryos, injection of 20 pg of *vegf*$_{121}$ mRNA caused expression of *flk1* throughout the vasculature, including the region of the PCV at 25 hpf (Figure 5E; see Supplemental Table S2). A similar effect was observed in *mib*^{ta52b} mutant embryos, as ectopic expression of *flk1* was noted following injection of 20 pg of mRNA encoding Vegf$_{121}$ (Figure 5F; see Supplemental Table S2). Therefore, ectopic induction of *flk1* expression by Vegf does not require Notch activity.

Despite being responsive to Vegf$_{121}$, *mib*^{ta52b} mutant embryos fail to express arterial markers, such as *notch5* and *ephrin-B2a* (Lawson et al., 2001). To determine whether *vegf* could bypass the requirement for Notch signaling, we injected mRNA encoding Vegf$_{121}$ into embryos derived from an incross of heterozygous *mib*^{ta52b} parents and assayed for artery marker gene expression at 25 hpf. Expression of *notch5* is normal in wild-type siblings injected with EGFP mRNA or left uninjected (Figure 5G; see Supplemental Table S2), while ectopic expression within the region of the PCV is noted in wild-type embryos injected with 20 pg of *vegf*$_{121}$ mRNA (Figure 5I; see Supplemental Table S2). Embryos mutant for *mib*^{ta52b} injected with EGFP or left uninjected fail to express *notch5* in the DA or neural tube (Figure 5H; see Supplemental Table S2). The majority of *mib*^{ta52b} mutant embryos fail to express *notch5* mRNA within the DA when injected with 20 pg of mRNA encoding Vegf$_{121}$ (Figure 5J; see Supplemental Table S2). *notch5* expression is apparent in a very small percentage of mutant embryos (Figure 5J, inset; see Supplemental Table S2). We have noted similar results in response to 50 pg of *vegf*$_{121}$ mRNA and when using *ephrin-B2a* as a probe (data not shown).

The failure of Vegf to induce arterial marker gene expression in *mib*^{ta52b} mutant embryos suggests that Notch signaling is required downstream of *vegf* to induce arterial differentiation. To determine whether activation of the Notch pathway is sufficient to rescue arterial differentiation in the absence of Vegf, we utilized a two-component transgenic system. Adult fish expressing the yeast transactivator GAL4 driven by the zebrafish heat shock protein 70 promoter [*TG(hsp70:gal4)*; Lawson et al., 2001; Scheer et al., 2001] were crossed with fish harboring a myc-tagged form of the zebrafish Notch1a intracellular domain driven by the upstream activating sequence [*TG(uas:myc-notch1a-intra)*; Scheer and Campos-Ortega, 1999], and embryos were injected with 10 ng of either control or Vegf morpholino. The embryos were allowed to develop and were heat-shocked and processed as described in Experimental Procedures. Embryos injected with 10 ng of control morpholino that did not express ectopic Notch1a-intra (determined by absence of immunostaining for the myc epitope) show the normal pattern of *notch5* (Figure 6A) and *ephrin-B2a* (Figure 6F) transcript within the DA, while embryos injected with Vegf morpholino exhibit reduction of arterial *notch5* (Figure 6B) and *ephrin-B2a* (Figure 6G) transcript. In contrast, embryos expressing exogenous Notch1a-intra (as indicated by the brown staining in Figures 6C–6E, 6H, and 6I) that had been injected with 10 ng control morpholino showed ectopic arterial marker gene expression. This observation is in contrast to our previous work (Lawson et al., 2001) and is likely due to a difference in timing following heat shock, as we have allowed a longer lag period to elapse in the experiments presented in this study (see Experimental Procedures). *notch5* appeared less responsive than *ephrin-B2a* to exogenous Notch1a-intra, with about 25% of embryos displaying detectable ectopic *notch5* expression confined to a few scattered cells within the PCV (Figures 6C and 6D; see Supplemental Table S3 at http://www.developmentalcell.com/cgi/content/full/3/1/127/DC1). In contrast, many more *ephrin-B2a*-positive cells were found within the PCV of all sibling embryos with Notch1a-intra expression (Figure 6H; see Supplemental Table S3). We also find that most embryos expressing Notch1a-intra that were injected with 10 ng of Vegf morpholino exhibit both *notch5* (Figure 6E; see Supplemental Table S3) and *ephrin-B2a* (Figure 6I; see Supplemental Table S3) within the DA, with ectopic PCV expression also apparent, indicating that activation of the Notch pathway in the absence of *vegf* signaling can rescue artery differentiation.

Discussion

Although anatomical differences between arteries and veins have long been known, it was not until recently that molecular markers could distinguish the endothelial cells lining these vessels within the circulatory system (Wang et al., 1998). This finding, together with the appearance of these markers before circulation, has led to the hypothesis that arterial-venous differentiation is governed by genetic mechanisms (Wang et al., 1998). In this study, we describe a cascade of well-known signaling factors (Figure 7) that is responsible for proper arterial-venous differentiation during embryonic vascular development.

Shh Regulates Arterial Differentiation by Inducing *vegf* Expression

We find that embryos lacking Shh activity fail to express *ephrin-B2a* within their blood vessels, similar to embryos lacking Vegf (this study) or Notch function (Lawson et al., 2001), while exogenous Shh induces ectopic formation of arteries within the trunk of zebrafish embryos. Other arterial markers, such as *deltaC* (Smithers et al., 2000) and *notch5*, respond in a similar fashion to

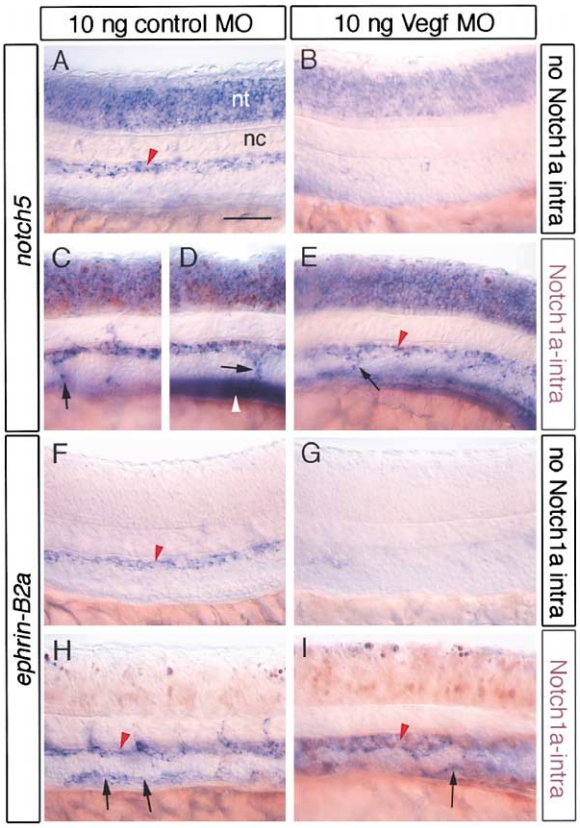

Figure 6. Notch Signaling Induces Arterial Differentiation Downstream of *vegf*

(A–I) All embryos were subject to heat shock as described in Experimental Procedures.

(A) Embryo without Notch1a-intra expression (as evidenced by the lack of myc-positive nuclei) injected with 10 ng control morpholino; *notch5* expression in the DA is indicated by red arrowhead; nc, notochord, nt, neural tube.

(B) Embryo without Notch1a-intra expression injected with 10 ng Vegf morpholino; note the lack of *notch5* DA expression.

(C and D) Images from the same embryo injected with 10 ng control morpholino and expressing Notch1a-intra (brown staining). Note ectopic *notch5*-positive cells in the PCV (indicated by black arrows); white arrowhead indicates expression in pronephric duct outside the plane of focus.

(E) Embryo injected with 10 ng Vegf morpholino and expressing Notch1a-intra; the red arrowhead indicates expression of *notch5* within the DA, and the black arrow shows region of ectopic PCV expression.

(F) Embryo not expressing Notch1a-intra and injected with 10 ng control morpholino with normal DA *ephrin-B2a* expression (red arrowhead).

(G) Embryo not expressing Notch1a-intra injected with 10 ng Vegf morpholino and lacking DA *ephrin-B2a* expression.

(H) Embryo expressing Notch1a-intra (brown staining) and injected with 10 ng control morpholino exhibits ectopic *ephrin-B2a* expression within the PCV (indicated by black arrows); normal DA position is indicated by red arrowhead.

(I) Notch1a-intra-positive embryo injected with 10 ng vegf morpholino. Note expression of *ephrin-B2a* within the DA (red arrowhead); ectopic PCV expression is also apparent (black arrow).

(A–I) Scale bar is 50 μm.

changes in Shh function (A.M.V., unpublished data), suggesting that Shh is a major upstream regulator of arterial differentiation. Our observations indicate that the role of Shh during this process is to induce expression of

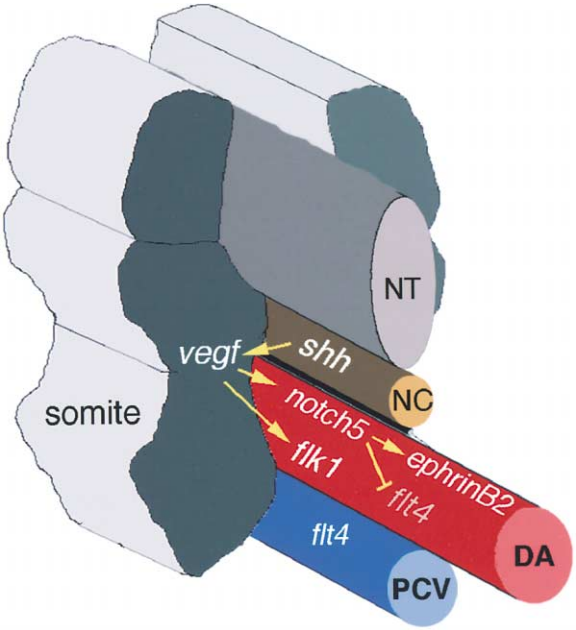

Figure 7. Model of Signaling Pathways Responsible for Arterial-Venous Differentiation

Diagram of a cross-section through a zebrafish trunk at approximately 24 hpf. NT, neural tube; NC, notcochord; DA, dorsal aorta; PCV, posterior cardinal vein.

vegf. Embryos lacking Shh activity do not express *vegf* mRNA within their somites, and injection of mRNA encoding Shh induces the expression of somitic *vegf*, which, in turn, acts on endothelial cells to drive arterial differentiation (Figure 7).

Although Shh acts upstream of Vegf to regulate arterial differentiation, we cannot rule out a direct role for Shh on endothelial cells during vascular development. The Shh receptor *patched1* is expressed in multiple cell types associated with blood vessels in mouse, including endothelial cells (Pola et al., 2001). Furthermore, angiogenic blood vessel growth exhibits qualitative differences in response to Vegf or Shh alone, possibly due to the ability of Shh to induce the expression of multiple endothelial growth factors, including *angiopoietin-1* and *-2* as well as *vegf* itself (Pola et al., 2001). This may explain the ability of exogenous Shh to induce the formation of ectopic lumenized vessels in zebrafish embryos, while Vegf is unable to elicit this effect. Furthermore, embryos lacking Shh activity often fail to form two distinct trunk axial vessels, a phenotype that is more severe than that observed in embryos lacking Vegf (this study) or Notch function (Lawson et al., 2001). These observations suggest that Shh may affect the morphogenesis of blood vessels through pathways other than those involving *vegf*. In addition, it remains a possibility that Shh plays a direct role in blood vessel development, although it does not appear to be a proximal factor in determining arterial cell fate.

Notch Signaling Acts Dowstream of Vegf to Induce Arterial Differentiation

Our experiments demonstrate that the Notch signaling pathway acts downstream of Vegf during arterial differentiation. We have previously shown that Notch activity

is necessary for the expression of some arterial markers (Lawson et al., 2001). We now demonstrate that Notch is also sufficient to induce ectopic artery marker gene expression in veins and is able to rescue *ephrin-B2a* and *notch5* expression in the absence of Vegf. In light of these findings and the observation that *flk1* displays artery-restricted expression even in the absence of Notch signaling, we find it of interest to note that expression of some arterial marker genes is also unaffected in *mib*^{ta52b} mutant embryos (Lawson et al., 2001). In addition, *ephrin-B2a* displays ectopic expression within the PCV more readily than *notch5* in response to Notch1a-intra, while rescue of *notch5* expression is often confined to the DA, with little ectopic expression apparent. Similarly, rescue of *ephrin-B2a* by Vegf in embryos lacking Shh often appears only within the region of the DA (for example, see Figures 3B and 3F). Taken together, these results suggest differential sensitivity of arterial genes to Notch and Vegf signaling and indicate the action of additional unknown factors that contribute to arterial differentiation and may confer spatial information upon the developing DA.

Vegf is known to elicit a wide range of responses in endothelial cells, including proliferation, migration, and survival (Ferrara, 1999). Our studies indicate that migration is not affected by the lack of Vegf, as endothelial cells move to the midline and are observed in the position of the DA in embryos injected with Vegf morpholinos. At this time, we cannot rule out the possibility that Vegf provides a proliferative or survival signal that is responsible for expanding a putative arterial progenitor cell population. However, if this were the central role of Vegf during this process, we would expect arterial marker gene expression at levels comparable to normal, but with a reduced number of positive cells within the DA. This does not appear to be the case. We often observe reduced expression of arterial markers throughout the DA at lower doses of Vegf morpholino (A.M.V. and N.D.L., unpublished data), suggesting that expression level of these genes, rather than cell number, is being affected. Thus, although we cannot dismiss other functions for Vegf at this time, we favor the possibility that it is providing a signal for the induction of genes that play an important downstream role in arterial differentiation.

Angioblasts within the zebrafish lateral mesoderm are restricted to an arterial or venous lineage (Zhong et al., 2001) by the 7–12 ss, suggesting that the specification of these cell fates occurs prior to this time. The components of the pathway we describe here (Figure 7) are appropriately expressed to play a role at this stage. *vegf* transcript is expressed within the somites in close proximity to *flk1*-positive angioblasts that are migrating to the midline at the 10 ss. In addition, *shh* is expressed within the notochord at this stage (Odenthal et al., 1996). It is unclear at this point whether Vegf is responsible for the intial specification of arterial progenitors or their subsequent differentiation. It is possible that the specification step occurs earlier, rendering lineage-restricted arterial progenitors more sensitive to the apparent inductive activity of Vegf. This may explain the differential effect of both Vegf and Notch activation on the arterial identity of endothelial cells noted above; in both cases, cells in the position of the DA, rather than the PCV, appear to respond more robustly to these factors. Alternatively, angioblasts most proximal to, or those first to migrate into, the midline may preferentially receive a Vegf signal and become specified as arterial progenitors. In either case, as lineage-restricted arterial progenitors migrate to the midline and come in proximity to the source of Vegf, they respond by inducing expression of *notch5*, which, in turn, leads to differentiation.

Recent studies in zebrafish indicate that the *hairy*-related transcription factor, *gridlock* (*grl*), is required for arterial differentiation. Zebrafish embryos mutant for *grl* exhibit defects in DA formation (Zhong et al., 2000), and reduction of Grl by morpholino oligonucleotides results in a loss of *ephrin-B2a* from the DA (Zhong et al., 2001). Furthermore, there is evidence supporting a role for *grl* downstream of the Notch pathway during this process, as the expression of both *grl* and its mouse homolog, HRT2, can be induced by Notch activity (Nakagawa et al., 2000; Zhong et al., 2001). These findings, as well as the similiarity of vascular defects observed in embryos lacking *grl* to those lacking Shh, Vegf ,or Notch activity, suggest that *grl* functions within the pathway described here (Figure 7) during arterial differentiation. In addition, activation of *notch5* or exogenous expression of *grl* can inhibit expression of the venous marker, *flt4* (Lawson et al., 2001; Zhong et al., 2001). Arterial expression of *flt4* is normally downregulated by 24 hpf (Thompson et al., 1998), a time at which *notch5* and *grl* are expressed in the DA (Lawson et al., 2001; Zhong et al., 2001). An obvious discrepancy in these observations is that *grl* is expressed at the 10 ss within the lateral mesoderm (Zhong et al., 2000), while *flt4* is expressed in all angioblasts beginning at this time and does not begin to exhibit downregulation within the developing DA until the 25 ss (Thompson et al., 1998). However, recent findings suggest that the levels of *grl* may be an important determinant of its function (Thurston and Yancopoulos, 2001; Zhong et al., 2001). Thus, during intial exposure to Vegf at the 10 ss, *grl* levels would be low within arterial progenitor cells, allowing expression of *flt4* in all angioblasts. As development proceeds, continuous exposure to Vegf would induce the Notch signaling pathway, and the subsquent increase in the level of *grl* in arterial progenitors would then lead to artery-specific gene expression and the downregulation of *flt4*.

The general pattern of the vertebrate circulatory system, as well as the basic signaling factors that regulate its development, have been conserved throughout evolution. It is now known that the early molecular dichotomy of arteries and veins is also conserved, as arteries in a number of vertebrates can be discerned from veins by virtue of specific molecular markers (Helbling et al., 2000; Lawson et al., 2001; Moyon et al., 2001; Wang et al., 1998). Recent work has demonstrated arterial-specific expression of *notch1*, *notch3*, and *notch4* transcripts in mouse embryos (Villa et al., 2001), consistent with our finding of DA-specific expression of *notch5* in zebrafish (Lawson et al., 2001), and suggests a similar role for these factors in the development of the mouse vasculature. Thus, it is likely that our present findings will be relevant to the process of arterial-venous differentiation during vascular development in other vertebrates as well as during neovascularization in adults. In addition, our results further underscore the importance

of establishing proper arterial and venous identity for normal vascular development to occur in vertebrates and demonstrate a complex genetic pathway that functions to establish these differences before the onset of circulation.

Experimental Procedures

Fish Lines

The following mutant lines were used in this study: *alb*^{b4}, *syu*^{t4} (Schauerte et al., 1998), *yot*^{ty119} (Karlstrom et al., 1999), and *mib*^{ta52b} (Jiang et al., 1996). Wild-type lines used were TL and EK. Fish handling and breeding were performed according to standard procedures (Westerfield, 1993). The *TG(hsp70:gal4)* (Lawson et al., 2001; Scheer et al., 2001) and *TG(uas:notch1a-intra)* (Scheer and Campos-Ortega, 1999) fish lines have been described elsewhere.

Whole-Mount In Situ Hybridization and Sectioning

Whole-mount in situ hybridization was performed as described elsewhere (Hauptmann and Gerster, 1994) using the following probes: *ephrin-B2a* (Lawson et al., 2001), *flk1* (Thompson et al., 1998), *flt4* (Thompson et al., 1998), *notch5* (Lawson et al., 2001), and *vegf* (see below). Where applicable, β-galactosidase activity was detected using X-Gal or Magenta-Gal (Biosynth) as chromogenic substrates. For analysis of *TG(hsp70:gal4);TG(uas:notch1a-intra)* embryos, *notch5* and *ephrin-B2a* expression were scored, and embryos were separated prior to antibody staining. Subsequently, detection of myc-tagged Notch1a-intra was performed using the 9E10 monoclonal antibody (Berkely Antibody Company, Berkeley, CA) as described elsewhere (Westerfield, 1993). Histology was performed using JB4 resin as described elsewhere (Westerfield, 1993). Images were acquired using a Zeiss Axiophot2 equipped with a Hamamatsu 4742-95 digital camera using OpenLab Software (Improvision) or a ProgRes mF digital camera (Jenoptik, Eching, Germany) using Adobe Photoshop.

Plasmids

Sequences encoding $Vegf_{121}$ and $Vegf_{165}$ isforms were amplified by PCR using oligonuclotides 5′-TATAGGATCCACAATCATGAACTTG GTTGTTTATTTGA (sense) and 5′-TATACTCGAGCTGCACTTAGTG TTTCGTTT (antisense) followed by TOPO cloning into pCRII-TOPO (Invitrogen, Carlsbad, CA). This plasmid was used to generate an antisense riboprobe for in situ hybridization, and sequences were confirmed by cycle sequencing using a Beckman CEQ2000. Vegf fragments were removed from pCRII by digesting with BamHI and XhoI and cloned into the pCS2+ vector (Rupp et al., 1994). pCS2+nβ-gal (Rupp et al., 1994) and p64T*shh* (Krauss et al., 1993) have been described elsewhere. Plasmids were linearized with Not I followed by phenol:chloroform extraction and precipitation with ammonium acetate and ethanol. Capped mRNA was transcribed from linearized templates using the mMessage mMachine kit (Ambion, Austin, TX).

Microinjections, Cyclopamine Treatment, and Heat Shock

RNA injections were performed according to standard protocols (Xu, 1999). Determination of *mib*^{ta52b} or *you*-type mutant embryos was based on morphology (for examples, see Lawson et al., 2001; van Eeden et al., 1996). Mutant *mib*^{ta52b} embryos display severe trunk curvature, while *you*-type mutants exhibit abnormally shaped somites. Neither of these phenotypes is apparent in wild-type microinjected embryos nor are they affected by *vegf* mRNA injection (data not shown). The sequences of the Morpholino oligonucleotides (GeneTools, Corvallis, OR) Vegf-A1, Vegf A-3, and Vegf-D4 used in this study are the same as those described elsewhere (Nasevicius et al., 2000). The Vegf-D4 morpholino (control) contains four base pair mismatches compared to the Vegf-A1 (Vegf) morpholino, and should not bind to the *vegf* transcript. Prior to injection, morpholinos were resuspended in nuclease-free water at 20 mg/ml. For injection, morpholinos were diluted in 0.5% phenol red, 240 mM KCl, and 40 mM HEPES (pH 7.4). Cyclopamine (Toronto Research Chemicals, North York, Ontario, Canada) was dissolved at 10 mM in 100% ethanol. Embryos were dechorionated by treating with pronase and then transferred to agarose-coated or glass dishes in egg water containing ethanol vehicle or 25 μM cyclopamine. Typically, embryos were placed in cyclopamine at approximately 90% epiboly and allowed to develop at 28.5°C until 24 hpf. For heat shock experiments, embryos derived from a cross of *TG(hsp70:gal4)* and *TG(uas:notch1a-intra)* adults were grown overnight at 24°C. The following morning, embryos (approximately the 16–18 ss) were transferred into 50 ml Falcon tubes (Becton Dickinson, Franklin Lakes, NJ) in 5 ml of egg water and placed in a 40°C water bath for 20 min. Embryos were then rinsed out of the conical tubes into 10 cm plastic dishes (Becton Dickinson) and incubated at 28.5°C for 7–8 hr. Embryos were then dechorionated by pronase treatment (Westerfield, 1993) and fixed in 4% paraformaldehyde.

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